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14. ABSTRACT
The goal of this study has been to develop Tobacco mosaic virus (TMV) and its derived virus-like-particle (VLP) as a universal platform for the assembly, display and integration of receptor layers onto sensitive and robust transducer systems for biological and chemical detections. Unique features of this system include its genetic programmability, self-assembly, multivalent display capabilities and ability to be integrated with a range of transducer surfaces, including chip based ELISA, optical disk resonators, impedance electrodes and square wave electrochemical platforms. Combined these investigations have provided the basic knowledge needed to develop

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Report Title

Virus Directed Assembly of Peptide Receptor Surfaces for Diverse Sensing Platforms and Applications

ABSTRACT

The goal of this study has been to develop Tobacco mosaic virus (TMV) and its derived virus-like-particle (VLP) as a universal platform for the assembly, display and integration of receptor layers onto sensitive and robust transducer systems for biological and chemical detections. Unique features of this system include its genetic programmability, self-assembly, multivalent display capabilities and ability to be integrated with a range of transducer surfaces, including chip based ELISA, optical disk resonators, impedance electrodes and square wave electrochemical platforms. Combined these investigations have provided the basic knowledge needed to develop highly flexible and cost-effective receptor layers that are compatible with a range of target molecules and can be easily integrated within highly sensitive micro- and nano-system sensing platforms.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received		Paper
08/21/2013	3.00	Ekaterina Pomerantseva, Markus Gnerlich, Adam Brown, Konstantinos Gerasopoulos, Matthew McCarthy, Xiao Z. Fan, James Culver, Reza Ghodssi. Tobacco mosaic virus: A biological building block for micro/nano/bio systems, Journal of Vacuum Science & Technology A: Vacuum, Surfaces, and Films, (08 2013): 0. doi: 10.1116/1.4816584
08/21/2013	4.00	Adam D. Brown, Lindsay Naves, Xiao Wang, Reza Ghodssi, James N. Culver. Carboxylate-Directed In Vivo Assembly of Virus-like Nanorods and Tubes for the Display of Functional Peptides and Residues, Biomacromolecules, (08 2013): 0. doi: 10.1021/bm400747k
TOTAL:	**2**	

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received		Paper
TOTAL:		

Number of Papers published in non peer-reviewed journals:

(c) Presentations

– F. Zang, X. Z. Fan, K. Gerasopoulos, L. Naves, J. N. Culver, and R. Ghodssi, "An Impedance Microsensor with Virus-like-particle Nanoreceptors for Accelarated ELISA-on-a-chip," Hilton Head Workshop 2014: A Solid-State Sensors, Actuators and Microsystems Workshop, Hilton Head, SC, June 8-12, 2014.

– X. Z. Fan, L. Naves, N. P. Siwak, A. Brown, J. Culver, and R. Ghodssi, "Virus-like-particles for next generation micro/nano-biosensors," Hilton Head Workshop 2014: A Solid-State Sensors, Actuators and Microsystems Workshop, Hilton Head, SC, June 8-12, 2014.

– F. Zang, X. Z. Fan, L. Naves, J. N. Culver, and R. Ghodssi, "Integration of a programmable bioreceptor with microsensor systems", Mid-Atlantic micro/nano alliance Spring 2014 Symposium: From Lab to Life: Field Based Applications of MEMS & NEMS, Baltimore, MD, March 10, 2014.

– F. Zang, X. Z. Fan, K. D. Gerasopoulos, H. Ben-Yoav, A. D. Brown, J. N. Culver, and R. Ghodssi, "Scale-down Effects: Towards Miniaturization of an Electrochemical Sensor using Biomolecules," IEEE Sensors 2013, pp. 1408-1411, Baltimore, MD, November 4-6, 2013.

– M. Gnerlich, H. Ben-Yoav, X. Z. Fan, F. Zang, E. Pomerantseva, and R. Ghodssi, "TMV Biofabrication Technology for Chemical/Biological Sensing and Engery Storage Applications", Enabling Nanofabrication for Rapid Innovation (ENRI 2013), Napa, CA, August 19, 2013

– F. Zang, H. Ben-Yoav, X. Z. Fan, A. D. Brown, D. L. Kelly, J. N. Culver, and R. Ghodssi, "Tobacco Mosaic Virus as Nanomaterials for Electrochemical Sensors," 10th International Workshop on Nanomechanical Sensing (NMC 2013), pp. 161-162, Stanford, CA, May 1-3, 2013.

– X. Z. Fan, L. Naves, N. P. Siwak, A. D. Brown, J. N. Culver, and R. Ghodssi, "A Novel Virus-like-particle (VLP) Bioreceptor Coated Optical Disk Resonator for Biosensing," 2013 MRS Spring Meeting, San Francisco, CA, April 1-5, 2013.

Number of Presentations: 7.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

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Received	Paper

TOTAL:

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(d) Manuscripts

Received		Paper
05/24/2013	2.00	Adam D Brown, Lindsay Naves, Xiao Wang, Reza Ghodssi, James N Culver. Carboxylate Directed In Vivo Assembly of Virus-Like Nanorods and Tubes for the Display of Functional Peptides and Residues , Biomacromolecules (05 2013)
08/23/2013	5.00	Faheng Zang, Xiao Fan, Konstantinos Gerasopoulos, Hadar Ben-Yoav, Adam Brown, James Culver, Reza Ghodssi. Scale-down Effects: Towards Miniaturization of an Electrochemical Sensor using Biomolecules, IEEE Sensors (06 2013)
TOTAL:		**2**

Number of Manuscripts:

Books

Received	Book
TOTAL:	

Received	Book Chapter
TOTAL:	

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Xiao Zhu Fan	0.50	
Adam Brown	1.00	
Faheng Zang	0.50	
FTE Equivalent:	**2.00**	
Total Number:	**3**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
James Culver	0.10	
FTE Equivalent:	**0.10**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME	
Xiao Zhu Fan (0.5)	
Total Number:	**1**

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See Attachment

Technology Transfer

Virus Directed Assembly of Peptide Receptor Surfaces for Diverse Sensing Platforms and Applications - W911NF1110138

Introduction: (Statement of problem studied)

Biologically derived peptides offer a unique source of recognition specificity for the detection and identification of a range of threats including explosives, toxins and viruses. However, incorporation of these peptides into robust sensor systems has been challenging due to a gap in our abilities to assemble and integrate these selective peptides into sensor devices. To address this challenge our team utilized the rod shaped macromolecular structure of the *Tobacco mosaic virus* (TMV) as a nano-scaffold to control the display, assembly and integration of receptor peptides within sensitive transducer systems. Progress toward these goals is outlined below for each proposed objective.

Summary of Most Important Results:

Objective 1. Virus directed nanotemplate assembly of receptor peptides.

We have recently created two additional virus-like particle (VLP) assemblies that permit the display of receptor peptides within the inner channel of the rod-shaped VLP. Previously we demonstrated the ability to attach flexible linkers and receptor peptides directly to the C-terminus of CP while maintaining its ability to form helical assemblies. For the first new inner channel display system we designed a VLP CP in which the entire long inner loop region (Δ-inner loop; residues 90 to 115) has been removed and replaced with a multi-cloning site, enabling the insertion of desired peptide sequences (Fig. 1). This inner loop modified VLP CP is still capable of helical rod assembly but now has an inner channel diameter of ~ 8 nm instead of the 4 nm produced by the unmodified VLP. We have already used this inner channel modified CP to display peptides that contain multiple histidine residues. The second inner channel system utilizes a circular permutant CP construct to reposition the C- and N-terminus to the inner channel (inner radius) of the helical rod (Fig. 1). We have recently confirmed the expression and helical assembly of the E50Q / D77N circular permutant CP within our bacterial expression system (Fig. 1). Testing both inner and outer radius display versions of specific receptor peptides is continuing as a means to identify optimal analyte binding.

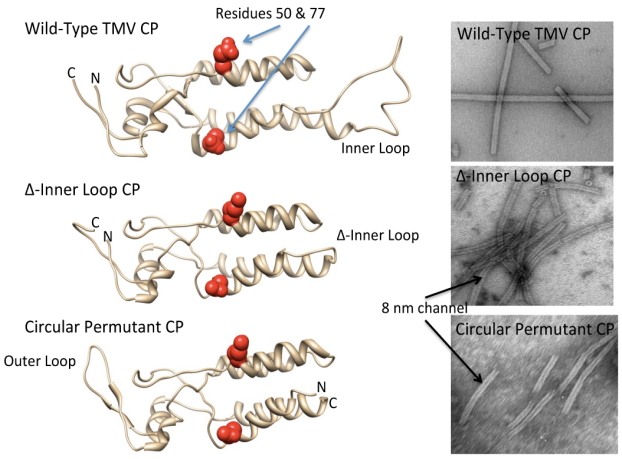

Figure 1. TMV CP modifications made and shown to assemble into helical nanorods via modification of the E50Q / D77N carboxylate pairs.

Objectives 2 and 3. Hierarchical patterning and assembly of virus receptor peptide nanoparticles and Assembly methods for the integration of patterned virus-based nanoparticles onto transducer surfaces. These objectives aim to identify methods to self-assemble and integrate TMV based receptor peptides into transducer systems. Information from these objectives will provide the base knowledge needed for the future development of robust microelectronic-based detection systems that utilize these strategies.

VLP as a free-floating binding agent for rapid TNT detection in solution. This task is aimed at developing a novel aqueous sensing method based on diffusion modulation of the target molecules through the use of TMV based VLPs displaying binding peptides selective for TNT.

The 2,4,6,-trinitrotoluene (TNT) is a widely used explosive and also a toxic chemical. There are growing needs for developing sensors that are capable of distinguishing and quantifying TNT in complex background environments, providing prompt feedback for environmental control and security purposes. Traditional electrochemical TNT sensors are widely used for the detection of TNT in solution; however, due to the electrochemical reduction of the nitro group, distinguishing TNT molecules from other aromatic compounds is difficult. Apart from electrochemical sensors, parallel research is concentrated on using surface immobilized functional layers of polymer, peptides or antibody bioreceptors in TNT sensors. But these methods need additional surface functionalization processes that slow down the speed of detection.

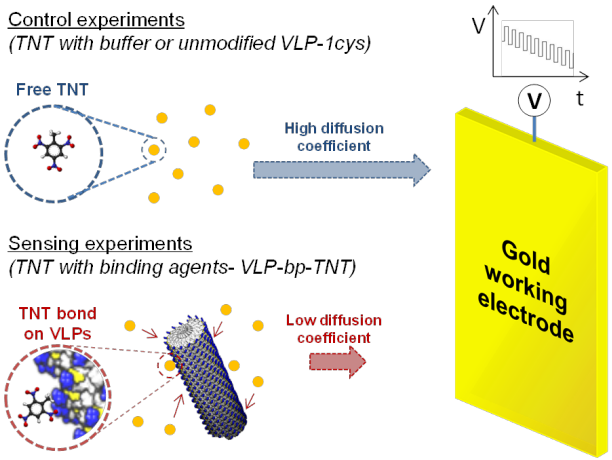

Figure 2. Diffusion modulations of TNT using VLP-bp-TNT binding agents

In this study, a novel electrochemical TNT sensor based on macromolecular binding agents was developed. It combines the fast response and high sensitivity of conventional electrochemical sensors with the selectivity of bioreceptors for rapid and label-free TNT detection. In the sensor, peptide modified TMV-VLPs are used as free-floating binding agents that selectively bind with TNT molecules in solution and lower the effective diffusion coefficient in the sensing system. A differential peak current due to the change in diffusion coefficient is therefore generated, and is directly proportional to the TNT concentration in the bulk solution (Fig. 2). Previously, our team has shown the capability of using the genetically modified TMV in the TNT sensing applications. However, limitations in virus based TMV yields hindered the development of this approach. Using the newly developed VLPs with TNT binding peptides (VLP-bp-TNT) eliminated the previous yield issues, producing concentrations of the VLP binding agent capable of higher differential signal level at given TNT concentration.

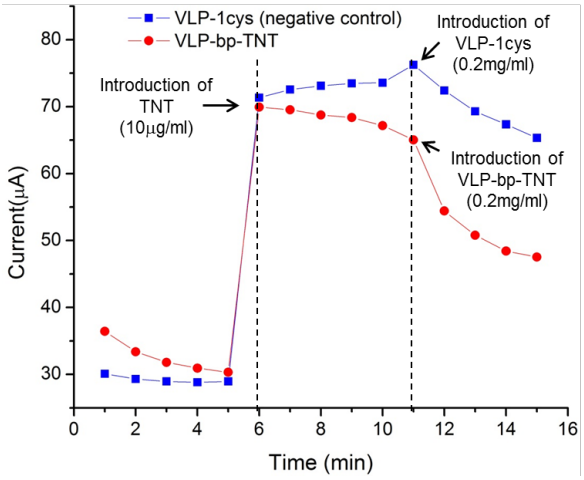

Figure 3. Dynamic responses of the peak current with sequential introduction of 10μg/ml TNT and 0.2mg/ml VLPs in solution

The dynamics of VLP binding with TNT were studied by monitoring the peak current response with sequential introduction of TNT and VLPs in solution (Fig. 3). The current decrease for the VLP-bp-TNT was significantly higher compared to the control VLP-1cys. In both cases, the currents stabilized after three minutes post introduction of the biomolecules. The instability of the current level in the first 3 minutes is a combination of biological binding and fluidic turbulence. Therefore, in later experiments, the TNT peak current was measured after 3 minutes of stabilization post introduction of VLPs.

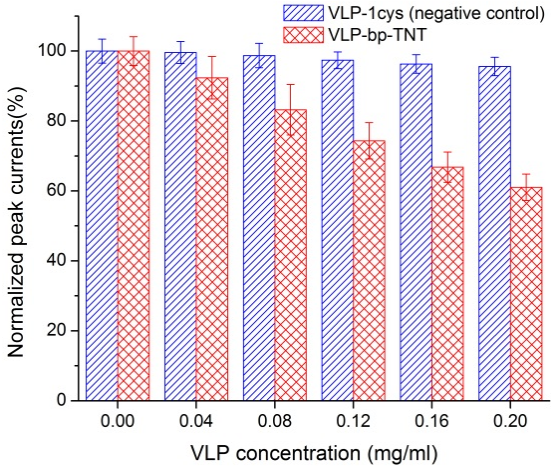

Figure 4. Normalized peak currents vs. VLP concentration obtained from the reduction of 10mg/ml TNT in solution

The effective diffusion coefficient of the electroactive molecules was controlled by the ratio of VLP bound and free TNTs in solution. The TNT peak current in the presence of increasing concentration of VLPs is shown in Figure 4. The current decreases to 61.0% as the concentration of binding agents increases to 0.2mg/ml. In the control experiment with VLP-1cys, the peak current is stable with only a 4.4% variation. The strong relation between the current level and binding agent concentration enables the sensitivity and resolution of the system to be adjusted through the differential current.

This work demonstrates the successful integration of genetically modified VLP binding agents in electrochemical sensors. More importantly, this novel sensing method can potentially be expanded to label-free and rapid detection of electroactive species that have great size contrast with biological binding agents.

Hierarchical patterning and assembly of virus receptor peptide nanoparticles. This approach is aimed at functionalizing the surface of optical resonant sensors using VLPs for the applications of highly sensitive and selective label-free biosensing.

The convergence of biology and micro/nanoscale technologies has allowed the construction of hybrid biodevices that have transformed chem-bio sensors. We have developed the VLP bioreceptor for microscale selective biosensing, whose selectivity may be tailored via genetic engineering. Each CP on the high-aspect ratio VLP nanorod was genetically mutated to allow for the self-assembly of VLPs onto surfaces via a cysteine (nCys) conjugation. An additional VLP construct with a second peptide sequence (cFlag) is expressed for specific binding of antibody (anti-Flag) to the CP (Figure 5).

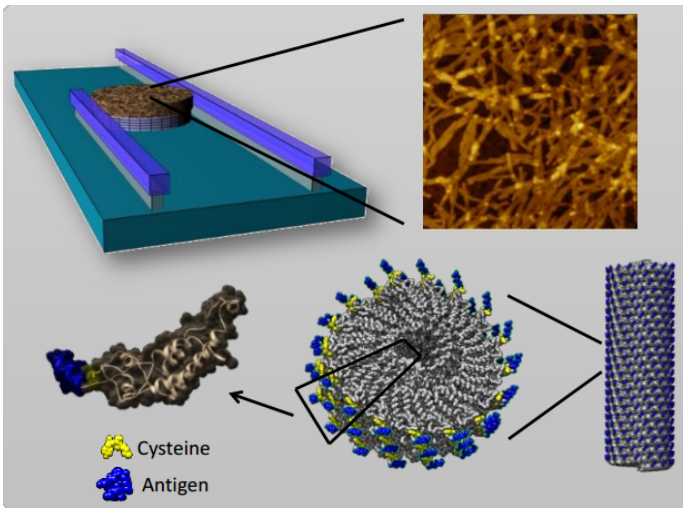

Figure 5. Schematic showing the expression of VLP on a microdisk resonator. The VLP nanostructure consists of identical CP subunits assembled into a helical formation. Each CP can be conjugated with a multitude of motifs, including cysteines (shown in yellow) and antigen peptides (shown in blue) used in this work.

An optical microdisk resonator is functionalized with the VLP receptor to conduct ELISA on-a-chip. The transducer is based on a change in the refractive index of the receptor layer upon the attachment of analytes. This change in cladding index will induce a measurable $\Delta\lambda o$. These types of sensors provide high sensitivity, and real-time measurement without the need for fluorescent or enzymatic labeling. The two types of VLPs were assembled on different optical microdisk resonators. VLP-nCys-cFlag was used to selectively bind to antibodies, and VLP-nCys was used as a negative control to monitor unspecific bindings. Both chips underwent identical ELISA, allowing for a sequential enzymatic binding of primary antibody, secondary antibody, and indicator assembly (Fig. 6). Label-free immunoassays were conducted by only introducing primary antibody. The selectivity of the bioreceptor layer was investigated against non-complementary antibodies (anti-HA and anti-His) and milk solution, which contains a host of proteins and salts that can contribute to non-specific binding.

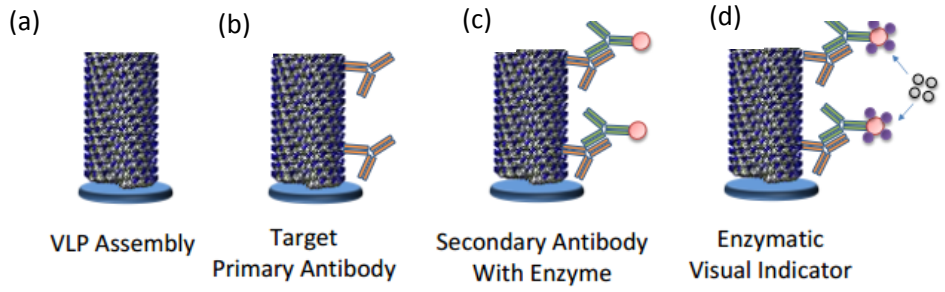

Figure 6. Sequential steps of the ELISA experimental procedure conducted.

As seen from Figure 7, the assembly of the VLP-nCys-cFlag and VLP-nCys on microdisk resonators induced a Δλo of 1.05±0.23 nm and 2.21±0.34 nm, respectively. The difference in wavelength shift is attributed to the difference in coverage density between the two VLP (confirmed by SEM). The ELISA procedure shifted the Δλo by +5.95nm (567%, with respect to receptor layer assembly) and -0.08nm (-4%) on VLP-nCys-cFlag and VLP-nCys coated sensor chips, respectively. The contrast in the Δλo indicates the selective binding of anti-Flag to the cFlag sites. Simplifying the ELISA process, label-free detection of only the anti-Flag showed a Δλo of +0.79nm (51%). VLP-nCys-cFlag coated sensor chips resulted in negative Δλo when exposed to the non-complementary antibodies, demonstrating its selectivity. The exposure to milk also showed no nonspecific binding to the receptor layer, Δλo=-0.03nm (-7.1%). The sensor was, however, able to detect the presence of anti-Flag, Δλo=+0.13nm (34.8%), when the milk solution was mixed with the antibody at 1:1000 dilution (Figure 8). The negative Δλo are all associated with the dehydration steps of the experimental procedure and were verified in an independent experiment.

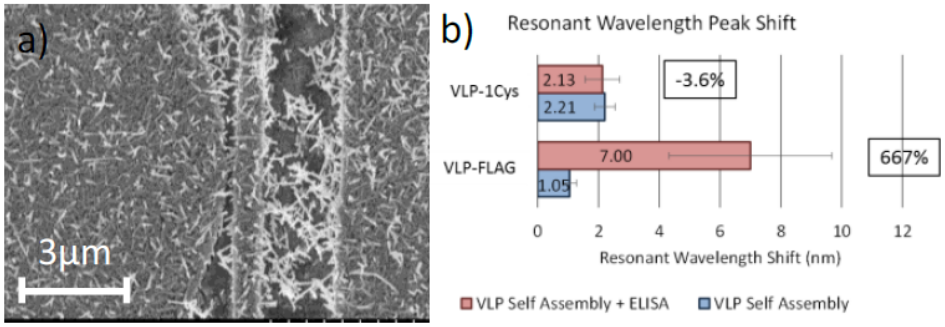

Figure 7. (a) A SEM image showing the assembly of VLPs on the surface of a micirodisk resonator (diameter: 20µm; thickness: 340nm; coupling gap: 300nm) (VLPs are metal coated for imaging). (b) Resonant wavelength shift of pre- and post-ELISA on VLPn1Cys and VLP-n1Cys-cFlag coated sensor chips.

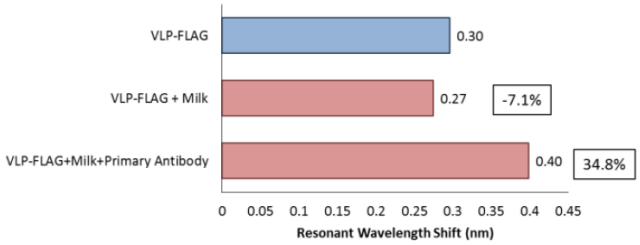

Figure 8. Resonant wavelength shift of sensor chip after submersion in milk, -7.1%, and milk with primary Flag antibody, +34.8%, demonstrating the high selectivity of VLP-n1Cys-cFlag.

The results presented here demonstrate the capability of a VLP-based receptor whose genetically programmable CPs can display a multitude of binding motifs on transducer surfaces for target analytes. The sensitivity of the platform enabled, for the first time, utilization of VLP for label-free detection and simplification of the standard ELISA process and conduct, decreasing time and cost of immunoassays and creating new sensing opportunities.

Impedimetric sensors for the real-time monitoring of VLP based bio-sensing. The goal of this task is to optimize the VLP assembly process on transducer surfaces using impedimetric microsensors in order to accelerate on-chip enzyme-linked immunosorbent assays (ELISA).

A selective and sensitive diagnosis of pathogens is a crucial step in identifying the source of disease and formulates the appropriate treatment strategy. Immunoassays such as sandwiched enzyme-linked immunosorbent assays (ELISA) are among the most effective and widely used methods to sense specific types of pathogens. The sensing efficacy of immunoassays is largely determined by the density and binding affinity of the immobilized sensing probes. Currently, one major problem associated with the most commonly used antibody sensing probes in immunoassays is their low capture efficiency on the immobilized surface. Because of this, surface functionalization is not maximized and the sensitivity of the immunoassay is not optimized. Besides, most surface functionalization procedures for immunoassays require several steps of chemical treatments on the surface. This prolongs the immunoassay, making it a time consuming and complex process. For these reasons, there is a need for development of sensing probes that are capable of functionalizing sensor surfaces rapidly and densely.

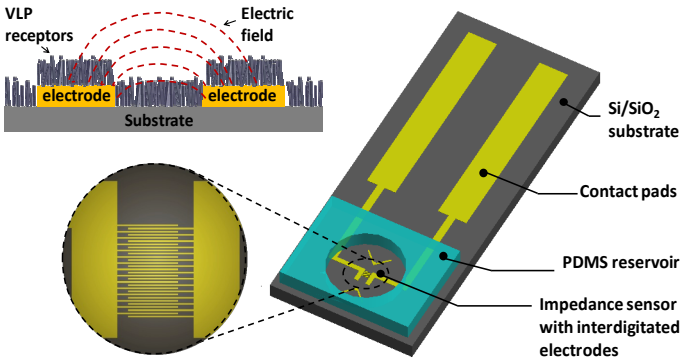

Figure 9. Schematic of the impedance sensor

In this work, we leverage the benefits of impedance sensors such as compatibility with liquid environments and high sensitivity to the surface capacitance/resistance change to monitor the nanoscale sensing probe functionalization in real-time and optimize on-chip ELISA. VLPs modified with cysteine groups and FLAG-tag peptides (VLP-FLAG) were used as sensing probes to anti-FLAG antibody to study the antigen-antibody interaction. The electrical impedance between the interdigitated microelectrodes is continuously monitored in the process of VLP-FLAG self-assembly in order to understand the dynamics of the VLP surface functionalization. Figure 9 shows a schematic of the impedance sensor which comprises gold interdigitated electrodes, contact pads and a PDMS reaction chamber. Impedance sensors with minimum feature size of 1μm were microfabricated.

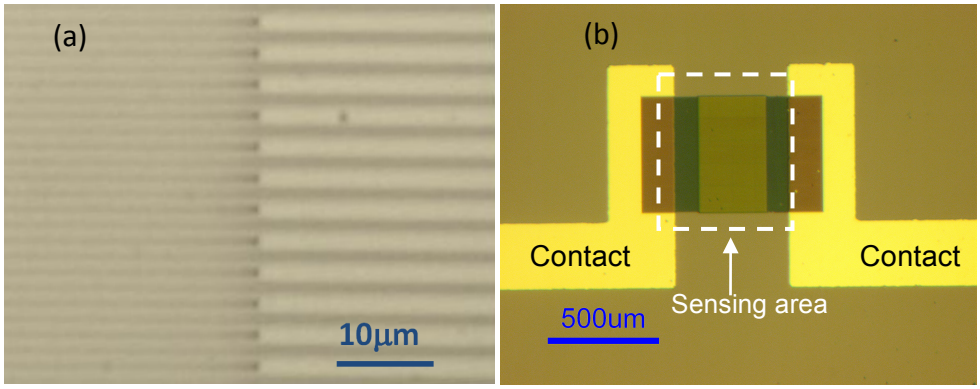

Figure 10: Optical images of (a) electrode features after e-beam lithography with PMMA e-beam resist and (b) the impedance sensor after fabrication

Figure 10a shows optical images of the 495 PMMA A10 resist after the e-beam lithography and the O2 plasma cleaning process using 40 watts of RF power for 16 seconds. The plasma cleaning helps to remove PMMA residues from e-beam lithography and development, which contributes to clean interdigitated features. The patterned interdigitated electrodes after e-beam deposition of Cr/Au and lift-off are shown in Figure 10b.

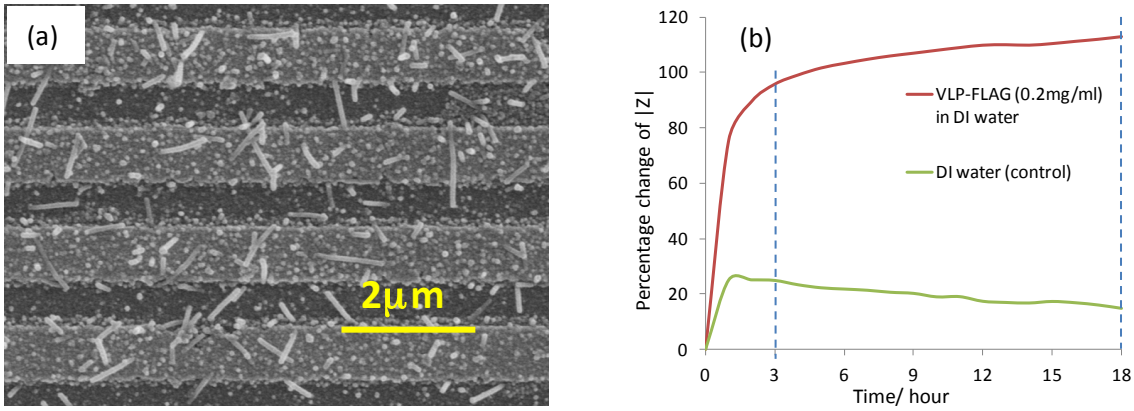

Figure 11: (a) SEM images of interdigitated electrodes after VLP-FLAG assembly in deionized water. (b) Percentage changes of impedance in the presence or absence of VLP-FLAG

The VLP-FLAG sensing probes suspended in the stock solution were added in the PDMS reaction chamber to functionalize the impedance sensor. The cysteine residue exposed at the terminals of the VLP-FLAG nanorods contributed to vertical self-assembly of VLPs on the electrode area. The time of self-assembly was varied from 0 to 18 hours in the study. The VLP assembly for 18 hours was previously used as a standard assembly process to get a uniform VLP functional coating. The surface morphology of the VLP functional layer in the interdigitated electrode sensing area after 18 hours of assembly was studied by scanning electron microscopy (SEM). A four-minute electroless plating of nickel was performed before SEM imaging to promote the conductivity of the surface. The SEM image in Figure 11 (a) shows the distribution of the VLP sensing probes on the sensing area of the impedance sensor after 18 hours of self-assembly. The self-assembled VLPs were observed both on the gold electrode and silicon oxide substrate. The impedance between interdigitated electrodes was monitored in real-time over the 18-

hour period to study the VLP assembly dynamics. DI water, which acts as the solvent for VLP, was used in the parallel control experiment to generate a baseline impedance response. The percentage changes in the amplitude of impedance at the frequency of 100Hz in the presence and absence of VLP probes are plotted in Figure 11(b). In the control experiment with DI water, the normalized impedance amplitude shows an increase of 25.1% in the first 1.5 hours and is stabilized at approximately 14.8% over the 18-hour period. The normalized impedance of the sensor in the presence of VLP-FLAG increases dramatically in the first 3 hours by 95.7%, after which it starts to saturate, gradually reaching up to 112.8% after 18 hours of assembly. This result indicated the optimized VLP assembly time is about 3 hours.

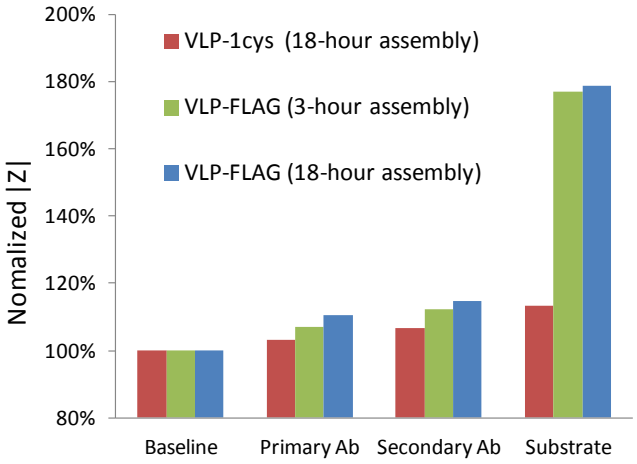

Figure 12. Normalized impedance of VLP-FLAG or unmodified VLP functionalized sensors during ELISA.

Figure 12 shows the normalized impedance amplitude during ELISA experiments. The baseline for each experiment was acquired based on the measured impedance after 30 minutes of 5% milk nonspecific blocking. The total normalized impedance for sensors functionalized for 3 hours and 18 hours using VLP-FLAG increased by 77.1% and 77.8%, respectively, indicating a similar biosensing efficacy in immunoassay. The significant change in impedance is due to the formation of insoluble precipitates that cover the sensing area and alter the surface morphology. In comparison, when VLP-1cys control probes are used, the impedance change is only 13.3%, which is attributed to non-specific binding. These results showed that the impedance sensors with reduced VLP self-assembly time (3 hours) can detect the presence of target antibody with high efficacy.

Thus, this study has contributed to optimizing the VLP assembly process and reducing the time by 6-fold compared to the regular 18-hour process.

Summary of final year accomplishments

Based on the results discussed in the previous sections, a summary of our major milestones during the final year of the project are listed below:

1) Development of VLP helical assemblies for the inner channel display of receptor peptides.

2) A novel TNT sensing method has been successfully developed based on the use of solution suspended VLPs as binding agents to selectively bind and measure TNT molecules in solution.

3) Optical ring resonators with VLP receptors were shown to perform label-free detection of target antibodies.

4) Impedimetric sensors were used for characterizing the optimized VLP assembly time on transducer surfaces and monitoring the immunoassay in real-time.